CellSafe

HiSense™
Mycoplasma PCR Detection Kit

Instruction Manual

Cat. No. HDEP-25, HDEP-50, HDEP-100

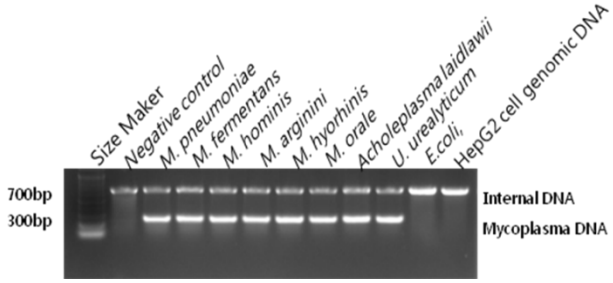
Research Use Only. Not for Use in Diagnostic Procedures.

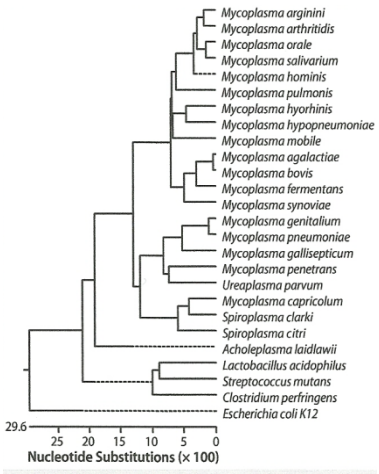
Introduction

HiSense™ Mycoplasma PCR Detection Kit utilizes the PCR, which is established as the method of choice for highest sensitivity in the detection of mycoplasma, ureaplasma and acholeplasma contamination in cell culture and other cell culture derived biologicals. The primers are specific to the highly conserved 16S ribosomal RNA sequences in the mycoplasma genome. This allows for detection of all mycoplasma species including *Acholeplasma laidlawii, M. arginini, M. fermentans, M. gallisepticum, M. genitalium, M. hominis, M hyorhinis, M. orale, M. pneumoniae, Spiroplasma citri* and *U. urealyticum*. All mycoplasma species including 8 genus and about 209 species can be detected simultaneously. The "European Pharmacopoeia" and Guideline of "Korea Ministry of Food and Drug Safety" (KMFDS) recommend checking for unspecific detection *of Clostridium, Lactobacilllus*, and *Streptococcus*. The 16S ribosomal RNA of other bacteria such as *E.coli, Clostridium, Lactobacillus, Sterptococcus*, and plant and animal cells is not amplified.

Kit Specificity

The *HiSense* Mycoplasma PCR Detection Kit detects *Mycoplasma* species simply, reliably, and rapidly. To detect the presence of these microorganisms, the assay uses the polymerase chain reaction (PCR) to amplify a target unique to a wide variety of mycoplasmas. The kit can detect more than 209 different *Mycoplasma species, including Acholeplasma laidlawii and Spiroplasma citri*. The kit does not detect other genera or cell-line DNA.





Kit Sensitivity

The sensitivity of the PCR using this kit is 1 to 10 copies of the target DNA per reaction. Sensitivity of the assay in real culture samples depends on the quality of the sample preparation.

Preventing Template Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for template preparation and PCR setup steps. Use the PCR grade water delivered with the kit, aerosol-preventive filter tips and gloves.

The 2X PCR master mix contains dUTP instead of dTTP. When dUTP replaces dTTP in PCR amplification, UNG treatment (Uracil-N-glycosylase, not provided in this kit) can prevent the subsequent reamplification of dU-containing PCR products. UNG acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites. When this strategy is put to use, carryover contamination will be eliminated while template DNA (DNA containing T) will be left intact.

Materials Provided

Materials Provided	Quantity	
	HDEP-50	HDEP-100
2x PCR Master Mix (Blue Cap)	750µl	1.5ml
Primer Mix (Red Cap)	100µl	200µl
Internal Amplification Control DNA (Orange cap)	100µl	200µl
Internal Amplification Control DNA for sample prep. (Violet cap)	1ml	2ml
Positive Control DNA (Yellow Cap)	25µl	50µl
DNase Free Water (White Cap)	600µl	1.2ml

Storage Conditions

Upon receipt, store at -20℃.

Note:

1) Repeat thawing reduces quality of product.

2) If frequent freeze and thaw is needed, aliquot the products and use in order.

Expiration Date

12 Months

Note: Please check the label on the product for details.

Test Protocol

Prepare the template (Sample)

Samples should be derived from cultures which are at 90-100% confluence. Penicillin and streptomycin in the culture media do not inhibit PCR or affect test sensitivity. To avoid false positive results, we recommend the use of the PCR grade water delivered with the kit, aerosol-preventive filter tips and gloves.

The preparation of sample screening:

Sample preparation from cell culture media

1) 1.2ml liquid supernatant of the sample is transferred into a 1.5ml tube and centrifuged (5 minutes, 1,000 rpm) to sediment cell debris.
2) 1ml of the supernatant is transferred into a new 1.5ml tube.
3) Centrifuged (10 minutes, 13,000 rpm) to sediment mycoplasmas.
4) Discard supernatant and wash the pellet once with 1ml of PBS. Repeat step 3).
5) Discard supernatant and add 50µl DNase free water or TE buffer to the pellet.
6) Heat the samples at 98℃ for 10min, and vortex for 5~10 sec. Then, centrifuge for 5 min at 12,000 rpm with a microcentrifuge. (*Caution!! Be careful when you heat the sample at 98℃. Heating it in PCR machine with heating cover is recommended.*)
7) Transfer the heated supernatant to a fresh tube. This supernatant will be used as the template in the PCR. Take 1~5µl supernatant as template for PCR reaction.
8) If the template contains PCR inhibition materials, the DNA can be purified with a commercial extraction kit.

The preparation of sample for EP 2.6.7 Guideline:

Genomic DNA extraction

*DNA was isolated using a commercial kit, DNeasy® Blood & Tissue Kit (Cat# 69504, Qiagen, Valencia, CA) following the procedure provided by the vendor

Preparation

All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
PBS is required for use in step 2. Buffer ATL is not required in this protocol.

Things to do before starting

Buffer AL may form a precipitate upon storage. If necessary, warm to 56°C until the precipitate has fully dissolved.
Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 4.

Procedure

1) **Collect 1ml cell culture ($5x10^5$~$1x10^6$ cells/ml) to a tube. Centrifuge for 10 min at 15,000rpm.**
   When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube.
2) **Decant the supernatant and resuspend the pellet in 200µl PBS.**
3) **Add 20µl proteinase K and 20µl Internal Control DNA*. Continue with step 4.**
   *The Internal Control DNA for sample prep. of the *HiSense* kit is used to verify the DNA extraction step as well.
4) **Add 200µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.**

It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

5) **Add 200µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.**
It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.
6) **Pipet the mixture from step 5 into the DNeasy Mini spin column placed in a 2ml collection tube. Centrifuge at 10,000 rpm for 1 min. Discard flow-through and collection tube.**
7) **Place the DNeasy Mini spin column in a new 2ml collection tube, add 500µl Buffer AW1, and centrifuge for 1 min 10,000 rpm. Discard flow-through and collection tube.**
8) **Place the DNeasy Mini spin column in a new 2ml collection tube, add 500µl Buffer AW2, and centrifuge for 1min at 20,000 x g (14,000 rpm).**
9) **Remove the 2ml collection tube solution and centrifuge at 14,000rpm for 3 minutes to dry the column membrane.**
It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.
Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

10) **Place the DNeasy Mini spin column in a clean 1.5ml or 2ml microcentrifuge tube, and pipet 50µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 2 min, and then centrifuge for 3 min at 10,000 rpm to elute.**

Prepare for PCR

1. Prepare the set of reactions listed in the following table. These include two types of control reactions: 1) **positive control** reaction(s) containing Mycoplasma positive control template DNA, and 2) **negative control** (no template).
(Caution!! Don't vigorous vortexing.)

Reaction Components	gDNA Sample Reaction	Cell Culture Media Reaction	Control Reactions	
			Positive Control (Control DNA)	Negative Control (No Template Control)
2x PCR Master Mix (Blue Cap)	15µl	15µl	15µl	15µl
Primer Mix (Red Cap)	2µl	2µl	2µl	2µl
Test Sample	5µl	1~5µl	-	-
Internal Amplification Control DNA (Orange cap)	-*	2ul	2µl	2µl
Positive Control DNA (Yellow Cap)	-	-	1µl	-
DNase Free Water (White Cap)	Up to 30µl			
Final volume	30µl	30µl	30µl	30µl

* In the sample reaction, internal amplification control DNA is not added separately because the sample already include internal amplification control DNA through the sample preparation of DNA extraction procedure.

2. Set up the PCR instrument to run the PCR cycling (amplification) program specified below.

Steps			Temp(℃)	Time
Pre Heat			94	5 min
PCR	40 Cycles	Denature	94	20 sec
		Anneal	60	30 sec
		Extend	72	30 sec

3. Apply 5~10μl ach of PCR products to the gel electrophoresis.

Results

When mycoplasma contamination exists, a band with around 250-270bp appears. An internal DNA band with around 700bp means the right performance of PCR reaction.

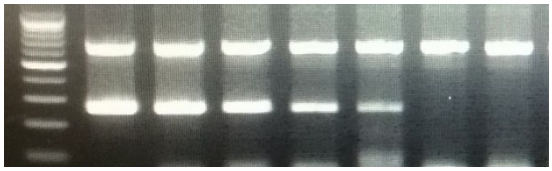


Fig. 1. Lane 1, 100bp DNA ladder; lane 2, 10pg *M. pneumoniae* DNA; lane 3, 1pg *M. pneumoniae* DNA; lane 4, 100fg *M. pneumoniae* DNA; lane 5, 10fg *M. pneumoniae* DNA; lane 6, 1fg *M. pneumoniae* DNA lane 7, 8, PCR reagent control (negative control)

Note:
1) Recommend to perform negative control without sample and positive control reaction by adding 1μl of mycoplasma control DNA.
2) If the PCR reaction is inhibited by high FBS concentration, the use of genomic DNA as a template may be helpful.
3) PCR inhibiting substances may accumulate in the medium of hybridoma cell. In this case, the use of diluted sample or genomic DNA as a template may be helpful.

Using the following table, determine whether the test cell culture is infected with *Mycoplasma*.

Mycoplasma Band	*Internal DNA Band*	*Interpretation*
Positive	*Positive*	*Mycoplasma contamination*
Positive	*Negative**	*Mycoplasma contamination*
Negative	*Positive*	*Mycoplasma non-contamination*
Negative	*Negative*	*PCR inhibition or Inadequate sample preparation*

* In case of severe mycoplasma contamination, internal DNA band can be not detected.